RESEARCH PATHOLOGY AND SPECIAL TECHNIQUES

ANNUAL REPORT

WILLIAM C. HALL

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Pathology Associates, Inc.
15 Worman's Mill Court
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The goals anticipated for the third year have been met. Immunoperoxidase procedures using the ABC and/or PAP methods have been completely developed for CCHF (KFD, RSSE) and Oropouche viruses, and partially developed for tularemia. Extensive support was provided for continuation of the work accomplished in previous years especially Ebolavirus in the only known outbreak in the United States, and in the dengue outbreak which occurred in Venezuela in late 1989 and early 1990. As a result of the latter, PAHO has requested that USAMRIID develop a workshop on these techniques for South American pathologists. The workshop is scheduled for October 1990. A training program and additional laboratory space has been established for performing the routine work of the Institute. A military technician is working in the Immunopathology Laboratory fulltime on the Ebola project; it is anticipated that technical personnel from PAI will need to continue at

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19. Abstract (continued)

USAMRIID on a fulltime basis. Collaboration with other Institute investigators from various Divisions has taken place on a routine basis. An adequate bank of resource materials has been archived to include animal tissues containing virus, adequate reagents, and aliquots of antibodies. Tissue preparation for immunoelectron microscopy has been completed. Work with the molecular probes has begun.

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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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BACKGROUND AND INTRODUCTION

The mission of the research at USAMRIID is multifaceted and has several major thrusts: 1) drug development for treatment and prevention of infection by potential BW agents, 2) toxin research leading to the discovery of methods to provide defense against a wide variety of toxins, and 3) rapid identification and diagnosis of disease agents, metabolites, and analogs in clinical specimens and collector samples.

By necessity, animal models are an integral part of the research dealing with BW agents. The use of the techniques described herein have already begun to enhance the BW defense program by providing thorough characterization of the disease models through localization of the agent and the host response against that agent in animal and human tissues. Moreover, the techniques are providing an alternative method for identifying the presence of a potential BW agent in tissues and bodily fluids and can be utilized as a rapid diagnostic procedure in some cases. Basic research on these agents requires that the infectious organism or toxin be localized in the various cells and tissues at different timepoints, and that the host response (cell type, antigenic cell markers, and humoral factors) be positively identified during the course of infection.

USAMRIID is a unique institution for performing research on exotic, potentially dangerous diseases because of its high containment facilities. Because of the potential danger of laboratory infection by these agents for studies performed outside the virus confinement areas (hot suites), the organism must be killed prior to the preparation of materials for pathological examination. Immunofluorescent studies are useful, but because the materials are not usually virus inactivated, they can be performed only within the hot suites. In addition, the resolution of many of the sections prepared in this manner for light microscopy is poor. Thus, alternative techniques which kill the agent and provide for excellent tissue detail are necessary for critical study of the disease processes.

Viral inactivation is usually accomplished by fixation of the tissues in formaldehyde, glutaraldehyde, or some other fixative adequate for inactivating the agent. Unfortunately, such fixatives generally alter the antigenicity of the virus being studied and often preclude the demonstration of the infectious agent in the cells and tissues of the infected host. Thus, it is difficult at times to determine whether lesions

observed by light and electron microscopy are caused primarily by the virus, other inoculated materials, adventitious agents, or host-mediated disease processes.

The purpose of this contract is: 1) to develop immunohistochemical or molecular probe methodologies to detect and localize infectious disease antigens and various host proteins in animal or human tissue specimens, 2) train USAMRIID personnel in the techniques and procedures that are developed, 3) collaborate with USAMRIID investigators in the preparation of publications and presentations, 4) develop and maintain an adequate bank of control and resource materials for the routine processing of animal model and human specimens, 5) bank aliquots of immunohistochemical reagents to be maintained under properly controlled conditions for future utilization by USAMRIID personnel, 6) modify immunolabelling procedures for use at the electron microscopy level, and 7) develop autoradiographic procedures for detection of viral agents for those cases in which immunolabelling at the light or electron microscopy level is inadequate.

MATERIALS AND METHODS

Basic Immunologic Procedures

Two primary methods were utilized for demonstrating antigens in tissue sections: avidin-biotin-peroxidase complex (ABC), and peroxidase-antiperoxidase (PAP) as previously described (*Lab. An. Sci* 34:261-263,1984). In addition, indirect labelling and fluorescent antibody techniques have been employed.

Reaction Product Substrates

A number of substrates for the immunoperoxidase and immunoalkaline phosphatase procedures were employed: 1) 3,3'-diaminobenzadine tetrahydrochloride (DAB), 2) DAB reacted with osmium tetroxide, and 6-bromo-2-hydroxy-3-naphthoic acid, an alkaline phosphatase (AP) substrate which produces a red reaction product insoluble in organic solvents. Permanent slides can be produced with all of the above substrates. The advantage of the AP label is that it produces a red reaction product which allows for differentiating antigen from endogenous pigments in the tissues. Moreover, endogenous peroxidase activity is often induced in macrophages and cannot be blocked with routine procedures using an immunoperoxidase system. This causes a problem in interpretation. The use of AP precludes this problem.

The AP procedure was especially useful in the Ebolavirus outbreak because of the presence of large quantities of iron-containing pigments in the tissues and the presence of excessive levels of peroxidase activity in macrophages. Use of immunoperoxidase gave questionable results.

Antigens

Viral antigens were derived from tissue culture or infected animal or human tissues. Each will be described in the appropriate results sections.

Fixation

Because of the universal use of formalin for fixation, attempts were always made to use this fixative where possible. Other fixatives used included formol sublimate (B5), acetone, IEM (2% paraformaldehyde and 0.1% glutaraldehyde), MBC (4% paraformaldehyde and 0.5% glutaraldehyde), Omni, PLP (periodate-lysine-paraformaldehyde), AMeX (acetone,methyl benzoate, paraformaldehyde) and zinc formalin. The use of MBC has given consistent results and it is used routinely where possible.

RESULTS

The major goal of the third year was to develop immunolabelling procedures for the detection of most of the remainder of viral antigens in fixed paraffin-embedded tissue sections. Procedures were completely worked out for CCHF, 2 members of the tick-borne encephalitis (TBE) group, and Oropouche virus. In addition, procedures were developed for the demonstration of tularemia antigen in human tissues. Attempts to demonstrate hepatitis A and simian hemorrhagic fever virus were unsuccessful. Further work was done to improve techniques or collaborate on studies with most of the agents worked on in the first two years of the contract.

Hantaan virus- Collaboration was carried on with Drs. John White, J. Dalrymple, and J. LeDuc. Studies were carried out in outbred strains of hamsters. Outbred female Syrian hamsters were infected with reference strain 76-118 of Hantaan virus. Subsequent to infection, they were bred in order to determine the mode of virus transmission to the offspring. Animals were killed approximately one month subsequent to parturition. Viral antigen was demonstrated in endothelium of a variety of tissues of the mothers but not the infants. This was shown for three litters. Thus, the mode of transmission of Hantaan among hamsters could not be determined in the short period of time of the study.

Additional suckling hamsters were inoculated with the virus. A small number of animals died spontaneously. Large amounts of Hantaan antigen were demonstrated in renal tubules of the dead hamsters indicating a probable means of viral spread to the environment. Lesions were not seen in the hamsters.

Human renal biopsies from Korea and autopsy material from Yugoslavia were examined for the presence of Hantaan virus antigen. Antigen was seen in endothelial cells and renal tubular epithelium in both instances. Lesions were extensive in the autopsy material.

Yellow Fever- Liver biopsy specimens were sent from Liberia by Dr. Mark Monson for yellow fever demonstration. This disease was not supposed to be present in Liberia. One sample was positive. Collaborative work has been done on yellow fever/dengue diagnosis from a variety of miscellaneous cases at the AFIP. This work has been done in collaboration with Dr. Kamal Ishak.

Studies on the relative pathogenicity of the 17D strain and a recombinent strain of yellow fever were undertaken in collaboration with Dr. Joel Dalrymple. Monkeys were inoculated intracerebrally and killed 7 days later in a neurovirulence assay. Lesions were more severe and antigen demonstration more intense in monkeys inoculated with the recombinent vaccine compared with the 17D strain. The neurovirulence assay appears to be a sensitive way to compare pathogenicity of yellow fever vaccines.

Dengue Viruses- USAMRIID was asked to collaborate with PAHO (Dr. Francisco Pinheiro) to diagnose individual dengue cases from an outbreak in Venezuela. Post mortem specimens from 19 cases were submitted for diagnosis. Using techniques established in the previous year, dengue was diagnosed in 4 of the 19 cases. By using a battery of monoclones, dengue strains 1 and 4 were eliminated and the disease narrowed to dengue 2 or 3. As a result of the efforts, PAHO has requested that USAMRIID present a workshop on yellow fever/dengue diagnosis using fixed tissues. The workshop is to take place in October in Belem, Brazil.

Ebola and Marburg Viruses- Work was carried out in collaboration with several individuals from Disease Assessment Division (Drs. Peters, Jahrling, Ksiazek, Johnson, Rollin), Pathology Division (Drs. Zack, Trotter, Jaax), and others. A large outbreak of Ebolavirus infection was diagnosed in a colony of monkeys from Reston, Va. Confirmation of the disease was made in the Immunohistochemistry Laboratory by use of monoclonal antibodies and by an antigen capture ELISA developed by Dr. T. Ksiazek. A cocktail of monoclones was utilized to react with all three strains of the

virus (Zaire, Sudan, and Reston). Of the antibodies used in the cocktail, only one (B-MD4-BD7-AE11) reacted strongly with all three virus strains. It also crossreacted with epithelium from renal tubules. The latter did not present a problem in interpretation. The monoclones M-BA6-C8A and M-DA1-A5-B11 reacted strongly with the Zaire strain, weakly with the Sudan strain, and equivocally with the Reston strain. DD4-AE-8A11 did not react with any of the strains. The results indicate definite differences among the strains.

Although Ebolavirus was demonstrated in a large percentage of the monkeys from the outbreak, simian hemorrhagic fever virus (SHF) was definitely demonstrated in a large number of the cases by virus isolation and electronmicroscopy. There is no current immunohistochemistry assay for SHF so that the Ebolavirus pathology is complicated by SHF which causes similar disease (disseminated intravascular coagulation). One aspect of the pathology of Ebolavirus that has been demonstrated with immunohistochemistry has been the presence of viral antigens in the respiratory tract (both upper and lower), the intestinal tract, and the urinary tract (urinary bladder and kidney). All of these point to various methods of elimination of the agent from the host which would contribute to spread of the virus in a facility. Some of these results have been published (*Lancet*, 335:502-05, 1990)

Punta Toro Virus- Work was completed on this agent and the results reported in the form of a manuscript (in press) in conjunction with Dr. George Anderson.

Rift Valley Fever- In conjunction with Dr. John Morrill of DAD Division and Dr. Ron Trotter of Pathology division, work was done to determine the distribution of viral antigen in aborted lambs, the placentas, and the infected ewes. In all lambs that aborted, antigen was widely distributed in the tissues and the placentas. In 2 lambs that were killed shortly after infection, antigen was demonstrated in the placentome but not in the fetal tissues. Hormonal assays for progesterone (Dr. Rippy) demonstrated a fall in maternal levels in all aborted ewes but not in those that were killed early. The results suggest that abortion is a direct result of placental/fetal infection.

Junin Virus- A large amount of work has been done on Junin virus in monkeys exposed by aerosol These studies are being reported on by Drs. Zack, Rippy, Ragland, and Kenyon. Immunohistochemistry techniques have played heavily in these studies.

Simian Immunodeficiency Virus (SIV)- Studies were continued from the previous year.

Congo Crimean Hemorrhagic Fever (CCHF)- Studies were done in scid mice reconstituted with human peripheral blood lymphocytes. Reconstituted and non-reconstituted mice were inoculated with CCHF and stained for viral antigen. Lesions and antigen were observed in the liver of reconstituted mice but not the non-reconstituted. The animal appears to be a potentially valid model to study CCHF. This work was done in collaboration with Dr. Topper.

Simian Hemorrhagic Fever (SHF)- Various antibodies have been assessed for demonstrating SHF antigen in fixed tissues. Serum from an African green monkey infected with SHF was fractionated and biotinylated. In addition, rabbit antibody against SHF was assessed. Neither antibody was successful in demonstrating SHF in infected monkey tissues.

Tick-Borne Encephalitis (TBE)- Pilot studies were done in monkeys to demonstrate Kyasanur Forest disease (KFD) and Russian Spring Summer encephalitis (RSSE). Both viral antigens were demonstrated, the former in the intestinal tract and the latter in the CNS. This work was done in collaboration with Drs. Rippy and Kenyon.

Hepatitis A Virus- Monkeys from the Reston outbreak of Ebolavirus had lesions consistent with viral hepatitis. Attempts to demonstrate the antigen using a polyclonal antibody failed. Further studies were not pursued.

Oropouche- Hamsters infected with Oropouche virus were stained for viral antigen in pilot studies. The antibody was titered and an ideal dilution determined. The pilot studies were positive for viral antigen. Additional studies are underway.

Ricin- Preliminary studies were undertaken to demonstrate ricin in exposed mouse tissues. Airway necrosis was observed but ricin could not be demonstrated using a polyclonal antibody. Further studies were done with frozen and formalin-fixed tissues sections where the sections were exposed to biotinylated ricin. The ricin was demonstrated on ciliated epithelial cells in the fixed tissues; background reactivity was extensive in the frozen sections. Work is still in progress to develop an assay for use in exposed tissues. These studies are difficult because of the very low tissues levels necessary to cause intoxication.

Tularemia and Cell Surface Markers- Tularemia antigen was demonstrated in the tissue of a biopsy specimen of an individual given the LVS strain of tularemia. Although background activity was high, tularemia antigen was demonstrated in macrophages in a granulomatous response in the skin. The reaction was characterized by infiltration of lymphocytes and macrophages into the dermis. The ratio of lymphocytes was approximately 2:1 CD8:CD4 cells indicating a strong response of the suppressor/effector lymphocyte population. Large numbers of macrophages were also demonstrated in the response.

A training program was established in the Immunopathology Laboratory wherein military and civilian personnel have been trained in immunoassay procedures. Moreover, an additional laboratory space has been established to perform much of the routine work. SOP's are available for all aspects of immunohistochemistry and technicians are performing these studies for various investigators on a regular basis.

Because of the large volume of work being generated by the Immunopathology Laboratory, personnel at PAI are continuing to work fulltime in the Laboratory in order to accomplish and exceed the anticipated goals. In addition, a fulltime military technician is working on the Ebolavirus project along with the contractor personnel from PAI. This is anticipated to continue along with other viral antigens.

Collaboration with many of the Institute investigators as evidenced in the foregoing has been continuous throughout the year. Strong ties have been made with some individuals in developing and designing various projects. These relationships are expected to continue.

Resource materials including aliquots of antibodies and tissues containing antigen have been established and will continue to enlarge.

Collaboration with Dr. John White and has taken place to develop immunoelectron microscopic demonstration of various antigens in tissue sections for Hantaan virus. The bulk of this work was accomplished by Dr. White.

To date, autoradiography has not been utilized as the immunological demonstration of the various antigens has been accomplished by means of immunoperoxidase techniques.

Work on the molecular probes has begun in a collaborative effort with Drs. P. Zack. Work is preliminary to date.

KEY WORDS

Immunchistochemistry
Hantaan virus
Dengue viruses
Yellow fever virus
Venezuelian encephalitis virus
Marburg virus
Ebola virus
Junin virus
Mouse hepatitis virus
Rift Valley fever
Machupo virus
Punta Toro virus
Lymphocytic choriomeningitis virus
Coxiella burnetii
Saxitoxin
Fibrinogen
Cell surface markers
Lymphocytes
Congo Crimean hemorrhagic fever
Simian hemorrhagic fever
Simian immunodeficiency virus
Oropouche
Tularemia
Tick-Borne encephalitis
Ricin
Hepatitis A